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GSK3 alpha and GSK3 beta are necessary for axon formation

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Abstract The mechanisms that underlie axon formation are still poorly understood. GSK3 has been recently implicated in establishing the axon and in its elongation. We have used four different GSK3 inhibitors to determine the role of GSK3 activity in hippocampal neurons at different periods of time. Inhibition of GSK3 activity impairs axon formation. The "critical period" of this activity of GSK3 is at least the first 24 h since afterwards the inhibition of GSK3 does not compromise the process of elongation, although it exacerbates axon branching. Moreover, interference RNAs impeding the expression of the GSK3 alpha or beta isoforms in hippocampal neurons prevents an axon from forming.

Keywords: Neuronal polarity; GSK3alpha; GSK3beta; Axon formation; GSK3 inhibitors; shRNA

1. Introduction

Neuronal polarization is a complex process that requires the morphological and functional differentiation of a somatodendritic and an axonal domain. The formation and outgrowth of the axon is one of the first events in developing neuronal polarity [1–3]. The morphological differentiation of cultured hippocampal neurons is produced when one of the neurites (Stage 2) becomes differentiated from the others and it grows faster to form the axon (Stage 3) [4,5]. The actin microfilaments and microtubule dynamics strongly influence the selection of one neurite as an axon [6–9]. The ensuing polarization and extension of the chosen neurite (the axon) may be controlled by the selective distribution of proteins such as Par-3, Par-6, APC, aPKC and Rap1b [10,11]. Finally, the mature axon is characterized by the selective localization of structural or functional proteins into specific domains like the axon initial segment (e.g. sodium channels [12]), conferring the neuron with its functional polarity.

The signals that underlie the decision of axon specification and outgrowth are still poorly understood and only recently has it been demonstrated that a signaling pathway controlled by PI3K may determine the appearance of an axon [10]. Indeed, the integrity and morphology of an axon may be reversed by inhibiting PI3K [13]. Hence, the hypothesis currently accepted is that many extracellular signals trigger a signaling cascade through PI3K, which regulates the dynamics of the actin and microtubule cytoskeleton. Downstream of the PI3-kinase, some elements have been described that may either control actin polymerization, such as Cdc42, or that influence MT dynamics such as Glycogen synthase kinase 3 (GSK3).

GSK3 is an ubiquitously distributed serine threonine kinase encoded by two different genes, alpha (α) and beta (β) [14,15]. Moreover, these genes encode a set of one alpha (α) and two beta (β1 and β2) splice variants [16,17]. GSK3 may be regulated by multiple mechanisms and its basal activity subjected to activation or inhibition [18–20]. The importance of this can be seen by the correlation of neuropathological processes such as AD or Ischemia with the deregulation of GSK3 [21]. In turn, GSK3 regulates microtubule growth and stability, phosphorylating many microtubule associated proteins such as tau, MAP1b and CRMP-2 or APC. Mutations or the absence of these proteins modifies the formation and growth of the axon. GSK3 phosphorylation of microtubule associated proteins such as MAP1B and tau appears to reduce their binding to microtubules, therefore maintaining a population of dynamically unstable microtubules [22]. Thus, GSK3 phosphorylation can render the microtubules more dynamic, favoring axon growth and supporting the idea that GSK3 activity is essential for axon outgrowth [23–27]. Accordingly, the MAP1B mutant mouse displays retarded axon outgrowth [28,29]. Hence, it seems that the establishment and outgrowth of the axon depends on the temporal and spatial regulation of GSK3 activity [26].

Contrasting results have been observed when GSK3 is inhibited pharmacologically. Thus, Jiang et al. [30] proposed that GSK3 inhibition induces multiple axon formation in neurons 6 days in vitro (DIV). In contrast, Shi et al. [31] had already proposed that inhibition of GSK3 during the first 2 DIV abolishes axon formation.

In the present work, we demonstrate that GSK3 activity is necessary for axon outgrowth. Both GSK3 isoforms are localized at the axon growth cone and their inactivation over 6 DIV abolish axon formation. However, inhibition of GSK3 after the axon has started to grow produces a substantial branching in the axon. Moreover, suppression of only GSK3α or only GSK3β using interference RNAs show that axon formation requires both GSK3α and GSK3β.

2. Materials and methods

2.1. Plasmids, inhibitors and antibodies

The GSK3 inhibitors used in this study were lithium chloride (Sigma), SB-216763, SB-415286 (Tocris) and AR-A014418 (kindly supplied by Dr. R. Bath from AstraZeneca) all of which were dissolved in DMSO.

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The GSK3 α and β RNA interference vectors, U6-GSK3αHP1 and U6-GSK3βHP1, were a generous gift from Dr. D.L. Turner (University of Michigan). The primary antibodies used were Tau-1 (1:1000, Sigma), SMI-31 (1:500, Affinity), 514 (1:400; polyclonal anti-MAP2, previously published [32]), PanNaCh (1:50, Sigma), tyrosinated tubulin (1:400, Sigma), ζPKC and APC (1:1000, Santa Cruz Biotechnology), GSK3β and GSK3α (1:200, Santa Cruz Biothecnology) and GSK3α/β (Biosource; 1:1000).

2.2. Cell culture

Primary hippocampal neurons were prepared from E17 mice. Hippocampi were digested with 0.25% trypsin, dissociated with a fire polished Pasteur pipette and plated on poly-L-lysine coated plates (1 mg/ml) at a density of 10000 cells/cm^2. After 2 h, the plating medium (MEM, 10% horse serum, glucose 0.6%) was replaced with Neurobasal medium supplemented with B27 and Glutamax-I. GSK3 inhibitors were added in fresh medium 4 h after plating and replaced every 3 days where necessary. Neuronal transfection was carried out 4 hours after plating using lipofectamine 2000 (9 μl, Invitrogen), 3 μg of U6-GSK3αHP1 or U6-GSK3βHP1 shRNA vectors and 1 μg of pEGFP-N1. The transfection mix was removed after 2 h and the neurons were washed and maintained for 3 DIV.

2.3. Immunofluorescence

Immunocytochemistry was performed on cultures of different ages following fixation of the neurons in 4% paraformaldehyde for 20 min. Non-specific binding was blocked with 0.22% gelatin and 0.1% Triton X-100 in 0.1 M phosphate buffer (PB). The cells were then incubated with primary antibodies for 1 h at room temperature, washed and incubated with Alexa conjugated secondary antibodies (1:1000). The coverslips were finally mounted using Fluoromount G (Southern Biotech) and images were obtained on a Zeiss confocal microscope. The results represent the means and S.D. of at least three independent experiments.

2.4. Western blot analysis

Hippocampal neurons were cultured for 2 days in poli-lysine coated plates at a density of 60000/cm^2, washed with PBS at 4 °C and homogenized in lysis buffer containing 100 mM NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA, 1% TX-100, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and leupeptin (Sigma), and 100 μM ortho-vanadate. The extracts were cleared by centrifugation at 15000 rpm for 15 min. The proteins of cell extracts were separated by 10% SDS–PAGE, transferred to nitrocellulose and the membrane was blocked with 5% non-fat milk before being probed with anti-actin, anti-tau1 and anti-PHF-1.

3. Results

3.1. Inhibition of GSK3 activity impairs axon formation in cultured hippocampal neurons

To test the role of GSK3 activity on axon formation in hippocampal neurons, we examined the effect of different inhibitors of this kinase including lithium chloride, SB-415286, SB-216763 and AR-A014418. The AR-A014418 does not significantly inhibit 27 other kinases demonstrating high specificity for GSK3 [33]. Hippocampal neurons were exposed to the inhibitors for 2 DIV or 6 DIV. The overall morphology of the neurons was examined using an antibody against tubulin, while the dendrites were identified by MAP2 staining. Axons were defined by their length as well as their recognition by the axonal markers SMI-31 (MAP1B-P) and tau-1 (Fig. 1).

Under control conditions, neurons developed one axon and several dendrites (86 ± 4%, n = 10). In contrast, an axon only developed in a small percentage of the neurons that were exposed to the GSK3 inhibitors for 2DIV (lithium chloride 10 mM, 7.28 ± 5.91%; SB-415286 50 μM, 18.5 ± 10.08%; AR-014418 20 μM, 12.44 ± 7.28%; n = 10; Fig. 1C and Supplementary Fig. 1S). This inhibition of axon formation mediated by the inhibitors of GSK3 was also dose-dependent (Fig. 1B). As a positive control of axon formation inhibition, we inhibited, in parallel, PI3K using LY294002 (50 μM). Under these conditions, only 13.82 ± 3.71% neurons developed an axon (n = 10; Fig. 1C).

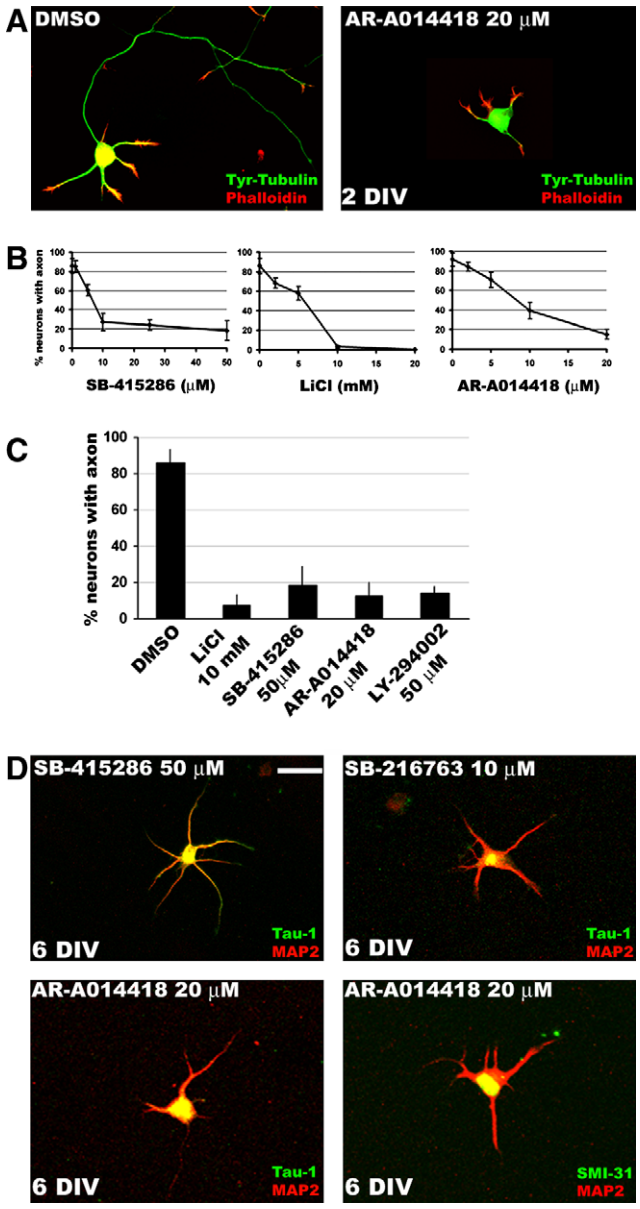

Fig. 1. Inhibition of GSK3 avoids axon formation in cultured hippocampal neurons. (A) After plating, hippocampal neurons were treated with the GSK3 inhibitor AR-A014418 and maintained in culture. After 2 DIV neurons were fixed and stained anti-tyrosinated-tubulin and phalloidin-Alexa 594. Neurons treated with DMSO developed axons that could be identified morphologically. In contrast, neurons treated for 48 h with 20 μM AR-A014418 were unable to develop axons. Scale Bar = 100 μm. (B) Dose-dependence effect of different GSK3 inhibitors in axon formation. (C) Percentage ± S.D. of neurons developing an axon after 48 h in the presence of GSK3 and PI3kinase inhibitors (n = 10). (D) Hippocampal neurons treated with GSK3 inhibitors for 6 DIV after plating do not form axons as shown by the absence of a tau-1 or SMI-31 positive processes. The somatodendritic compartment is identified by MAP2 staining. Scale Bar = 100 μm.

When neurons were treated with inhibitors for 6 DIV, a significant percentage of neurons were unable to develop an axon (30 ± 12%), while 12 ± 9% only developed a short axon, compared to control neurons (89.9 ± 5%, single axon). The effect of the inhibitors was specific to axon formation since inhibition of GSK3 did not affect the development of MAP2 positive dendrites, which did not differed significantly in length, morphology and number of dendrites from untreated neurons (Fig. 1A,D).

The inhibition of GSK3 was confirmed by Western blotting. Following GSK3 inhibition, there was a shift in Tau-1 mobility towards a lower molecular weight, at the same time as the PHF-1 almost completely disappeared. These changes reflected the failure of GSK3 to phosphorylate these proteins (Supplementary Fig. 2S). The incapacity to detect SMI-31 immunoreactivity also indicated that GSK3 activity had been completely inhibited (Fig. 1D), as this epitope is dependent on MAP1B phosphorylation by GSK3 at this neuronal stage.

To determine whether the short neurites extended, when GSK3 is inhibited, retain the potential to form axons, we removed the inhibitor and analyzed the capacity of neurons to form an axon under these conditions. After exposure to GSK3 inhibitors for 2 days (Fig. 2B versus A), the neurons were washed and grown in permissive conditions for a further 24 h (Fig. 2C). Irrespective of the GSK3 inhibitor used, around 75 ± 5 % ($n = 3$) of the neurons were able to recover from the inhibition of GSK3 and they grew an axon (Fig. 2C).

3.2. Axon formation requires GSK3 activity during the first 24 h

In order to asses the function of GSK-3 in axon establishment or axon elongation, we inhibited GSK3 in cultured hippocampal neurons only the first 24 h and let 5 more days in culture without inhibition. In this condition, most neurons developed a single axon after 6 DIV (90.4 ± 9%). However, these axons often presented an unconventional morphology, they were mostly branched (with multiple secondary and tertiary extensions) and in many cases they were curled (Fig. 3). To asses the possibility of multiple axons in GSK3 inhibited hippocampal neurons as proposed [30], we used an antibody that recognizes the axon initial segment [12] and is not related with GSK3 activity. The localization of sodium channels in the axon initial segment [12] permits the number of axons in each neuron to be clearly ascertained. However, in all cases only one axon initial segment could be detected (Fig. 3).

In order to determine whether the effect of GSK3 inhibitors on axon growth was dependent on a specific "pharmacological window", we permitted neurons an initial period of neurite growth and at different times thereafter, they were exposed to the inhibitors of GSK3. As a result, we found that the inhibition of GSK3 did not reduce axonal elongation when

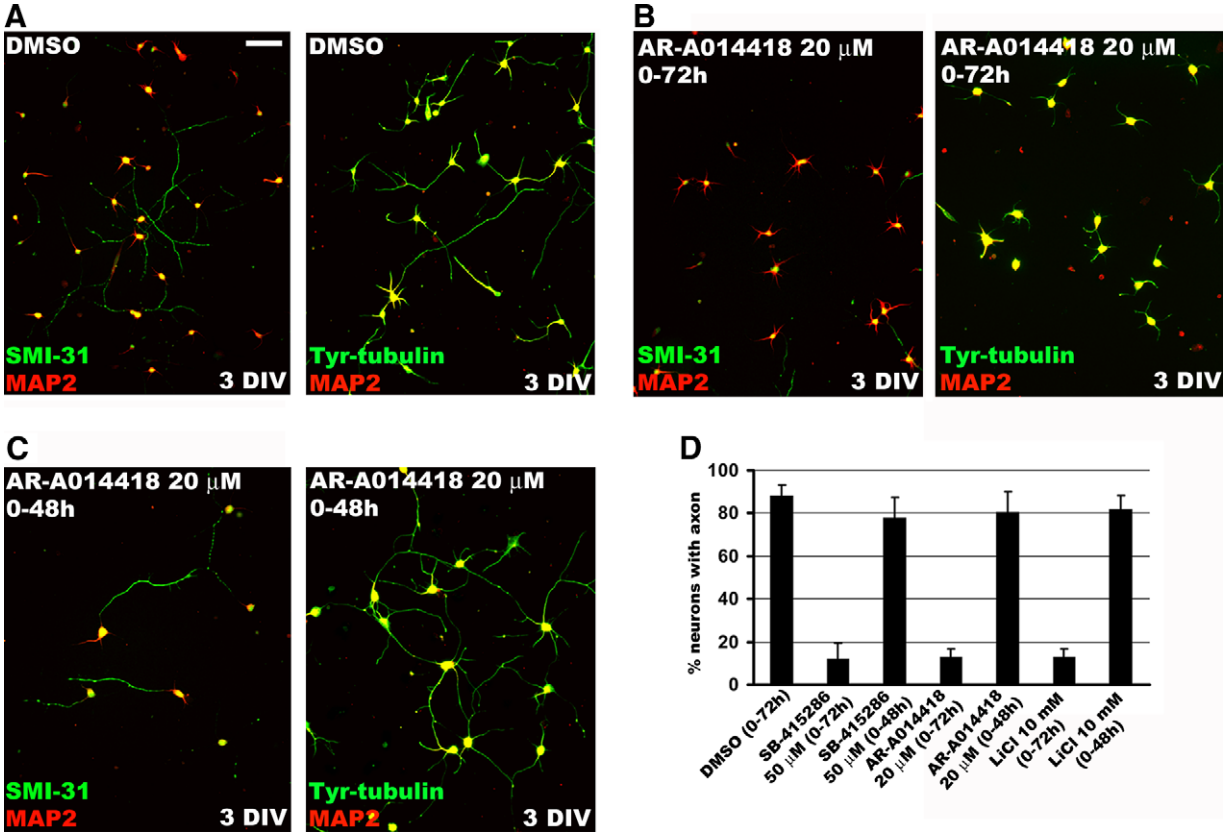

Fig. 2. The effect of GSK3 inhibition on axon formation is reversible. (A) Hippocampal neurons maintained for 3 DIV in the absence of GSK3 inhibitors form axons identified by the SMI-31 axonal marker and morphologically using anti-tyrosinated tubulin. (B) Neurons treated for 3 DIV with 20 μM AR-A014418 did not develop axons. (C) Neurons treated with GSK3 inhibitors for 2 DIV, washed and maintained one more day in culture without inhibition were able to develop SMI-31 processes (axons). (D) Percentage ± S.D. of neurons developing an axon in the absence or presence of AR-A014418, SB-415286 and Lithium Chloride for 3 DIV (0–72 h) or only the first 2 DIV (0–48 h) in three independent experiments. Scale Bar = 100 μm.

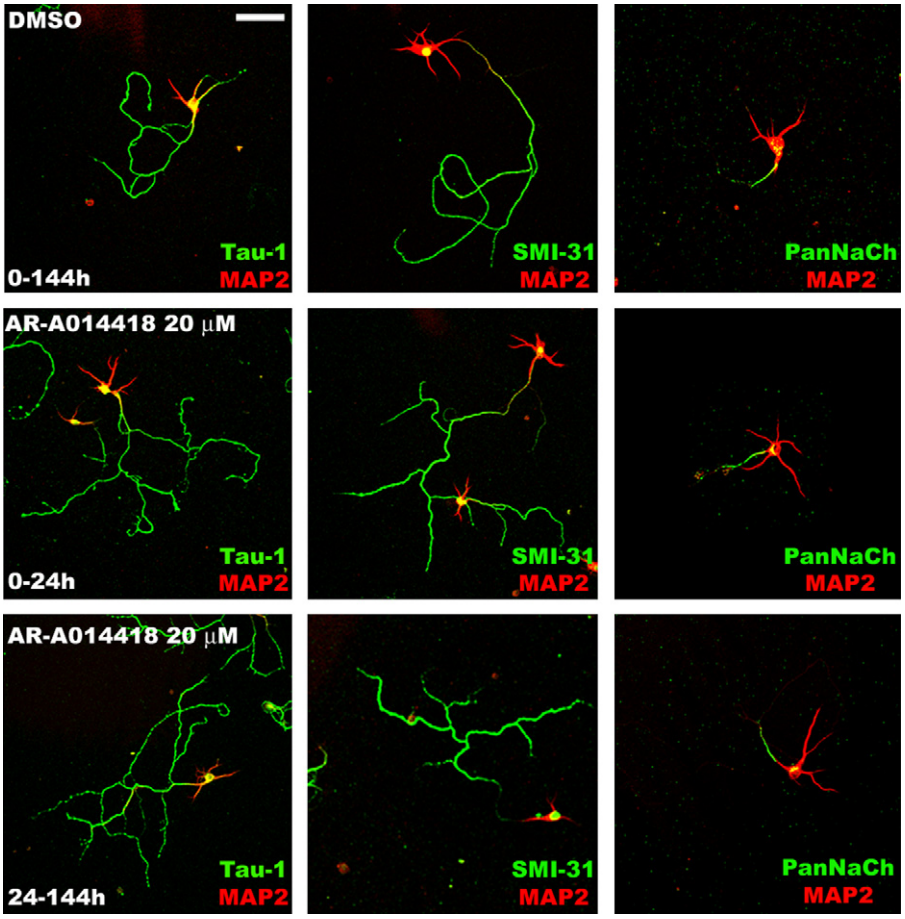

Fig. 3. Dual effects of GSK3 inhibition at different stages of axon development in hippocampal neurons. Neurons maintained in culture for 6 DIV in the absence of GSK3 inhibitors (upper row) were able to develop an axon that could be identified by tau-1, SMI-31 and the axon initial segment (AIS) marker PanNaCh (green). The somatodendritic domain was identified by MAP2 staining (red). Neurons treated with AR-A014418 20 μM only during the first day of culture (0–24 h, middle row) developed axons similar to control neurons but curled and ramified. (Bottom row) Inhibition of GSK-3 for 5 DIV after the first day in culture (24–144 h) did not affect axon formation and resulted in a pronounced axonal branching. In all cases only one axon per neuron was observed as indicated by the number of axon initial segments (right column). Scale Bar = 100 μm.

hippocampal neurons were initially maintained for one, two or three days under normal culture conditions (Fig. 3 and Supplementary Fig. 3S). In all cases, almost all neurons developed one axon (82 ± 12%, $n = 3$), as seen by the presence of only one axon initial segment. However, the axonal morphology was not totally wild-type as multiple ramifications and branching could be observed (Fig. 3 and Supplementary Fig. 3S).

3.3. GSK3α and GSK3β are localized at the growth cone of developing axons

The results obtained by inhibition of GSK3 show that GSK3 activity is necessary for axon formation, but also plays a role in the further axon development. This activity may control the function of other proteins located in the axon growth cone, such as APC or ζPKC. However, the inhibitors do not discriminate between GSK3 isoforms, alpha and beta. In order to asses the role of each isoform of GSK3, we immunostained 2 DIV cultured hippocampal neurons with antibodies that recognize specifically GSK3α or GSK3β. As shown in Fig. 4, GSK-3α and GSK-3β are localized in the soma and concentrated in the axon growth cone, but not in the growth cone of other neurites. Moreover, this localization resembles that of APC and ζPKC. When neurons were treated with GSK-3 inhibitors, axons did not developed and GSK-3α, GSK-3β, APC and ζPKC stainings were homogeneously distributed in the neuron without any concentration in growth cones.

3.4. Both GSK3 α and β are necessary for axon establishment

We have shown that the pharmacological inhibition of GSK3 with different compounds prevented the establishment of a polarized axon and that GSK3 isoforms, alpha and beta are present in the growth cone of a developing axon. These results indicate that GSK3α and/or GSK-3beta play a role in axon formation. However, we cannot conclude if only one or both GSK3 isoforms are responsible for axon formation. To extend this analysis further, we have used a genetic approach to interfere with GSK3 alpha or GSK3 beta expression. We performed RNA interference experiments with vectors that specifically interfere with the expression of GSK3α or GSK3β [34]. These vectors were first tested in N2A cells confirming that the expression of each isoform was suppressed by about 70% in accordance with earlier results (Fig. 5A). Subsequently, neurons were transfected with the GSK3α or GSK3β interference vectors in combination with a pEGFP-N1 vector, while hippocampal neurons transfected with pEGFP-N1 alone served as controls (Fig. 5B). Neurons were maintained in cul-

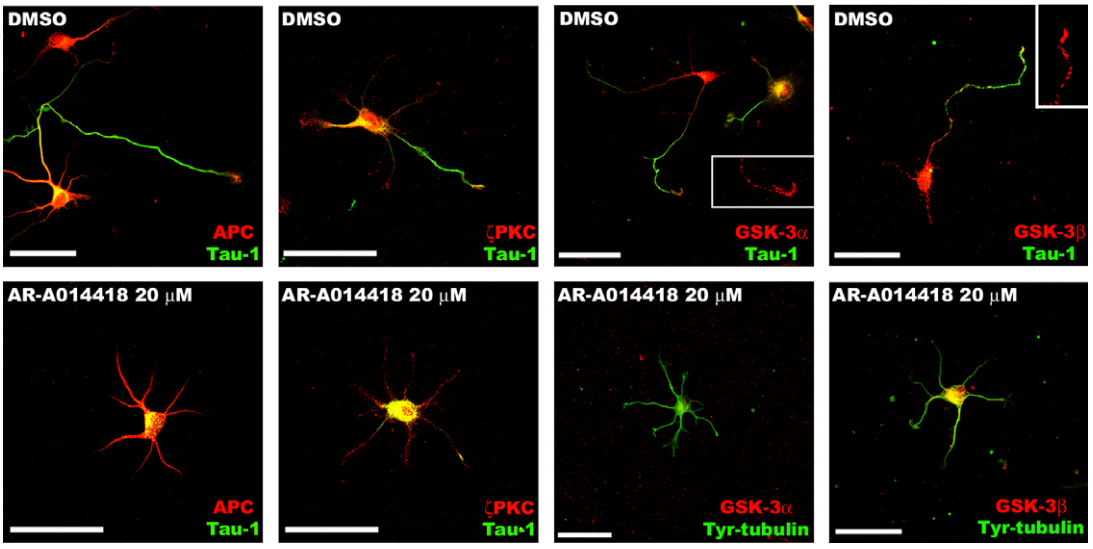

Fig. 4. GSK3 alpha and GSK3 beta are localized at the axon growth cone. Hippocampal neurons were grown for 48 h in the absence or presence of the GSK-3 inhibitor AR-A014418. Neurons were immunostained with antibodies against APC, ζPKC, GSK3α and GSK3β. Axons were identified by tau-1 staining. The images show that all four proteins are concentrated at the axon growth cone in control conditions. In the presence of GSK-3 inhibitor, neurons did not form an axon and all four proteins were distributed homogeneously in the soma and neurites. Scale Bar = 50 μm.

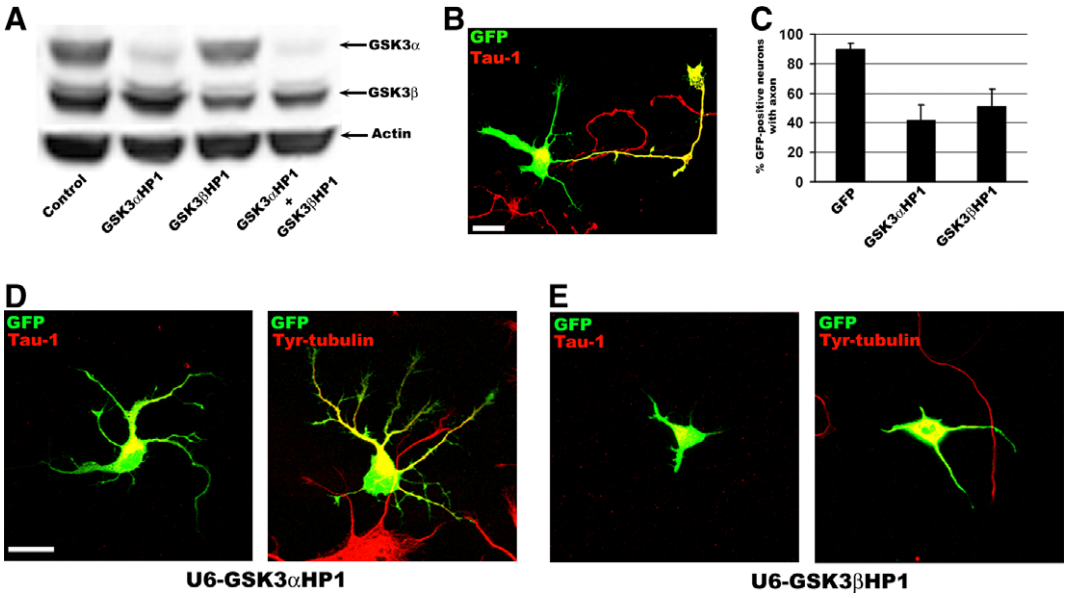

Fig. 5. Suppression of GSK3α or GSK3β expression impairs axon formation. (A) N2A cells were transfected with U6-GSK3αHP1 or U6-GSK3βHP1 RNA interference vectors and maintained for 3 DIV. Both vectors were able to reduce a 70% the expression of GSK3α or GSK3β, respectively. (C) These vectors were co-transfected with GFP in hippocampal neurons 4 h after plating. Only 42% of the neurons co-transfected with GFP and U6-GSK3αHP1 developed axons, and 51% when U6-GSK3βHP1 was transfected. In contrast, 89% of control neurons (B), transfected only with GFP, were able to develop an axon after 3 DIV. (D, E) Neurons transfected with U6-GSK3αHP1 or U6-GSK3βHP1 were stained with the axonal marker tau-1 or tyrosinated-tubulin in combination with GFP fluorescence. Neurons transfected with the RNA interference vector do not present any tau-1 positive process, neither a longer process identifiable in the tubulin staining. Scale Bar = 25 μm.

ture for 3 DIV after transfection, by which time an axon had fully developed in the control neurons, and the expression of different axonal and somatodendritic markers was assessed (Fig. 5B).

Suppression of GSK3α impaired the establishment of the axon, and only 42% ± 10% ($n = 4$) of the GFP positive neurons formed an axon (Fig. 5C). Similar results were obtained when GSK3β was suppressed (51 ± 12%, $n = 4$). Moreover, GSK3β seemed to be more important than GSK3α in the development of dendrites, since neurons transfected with GSK3α-HP1 interference RNA developed a more robust dendritic arbor (Fig. 5D) than those transfected with GSK3β-HP1 (Fig. 5E). Thus, while it has been proposed that GSK3β is the isoform that is principally involved in establishing the axon and in its elongation, the results presented here show that both isoforms are necessary to establish an axon.

4. Discussion

The establishment of neuronal polarity and axon outgrowth is a highly regulated and not well-known phenomenon. One of the signaling pathways that regulate the axon formation is the pathway of PI3-kinase. One of the enzymes controlled by PI3-kinase is GSK3, which is thought to participate in many pathways that control morphogenesis, synaptogenesis [35] or survival [36,37]. GSK3 is a widely distributed serine–threonine kinase encoded by two different genes, GSK3α and β [14,15]. Moreover, the developmental profile of GSK3α and β expression is different and specifically, the expression of the β isoform is down-regulated after birth.

Our results show that GSK3 activity is necessary in hippocampal neurons to establish an axon and maintained inhibition of GSK3 prevents axon formation. However, inhibition of GSK3, once the axon has started to elongate, produces axonal branching. Even more, this is the first time showing that the establishment of an axon requires both GSK3 isoforms, GSK3α and GSK3β, which are differentially concentrated along the axon.

4.1. GSK3 in neuronal polarity

Axon outgrowth and elongation is dependent on actin and microtubule cytoskeleton (MT) dynamics, as well as on the trafficking machinery that transports membrane components and proteins to the growing axon. The disruption of the actin cytoskeleton with cytochalasin D can affect the fate of neurites and it leads to the production of multiple axons [8]. After disrupting the actin cytoskeleton, elongation is achieved through the polarized growth of microtubules. The dynamics and the stability of microtubules are regulated by microtubule associated proteins (MAPs), which in turn are mainly regulated by phosphorylation. Indeed, the elimination of some of these proteins, such as tau or MAP1B using antisense oligonucleotides prevents axon initiation and elongation [38]. Accordingly, hippocampal neurons from MAP1B mutant mouse display impaired axonogenesis [39].

In general, GSK3 phosphorylates MAPs such as MAP1B or tau, making microtubules more unstable and dynamic. However, this phosphorylation appears not to be homogeneous throughout the axon and for that reason, tau-1 or MAP1B-SMI31 staining appears in a gradient in this structure. Thus, the normal growth of an axon appears to require a certain level of GSK3 activity [26,40,41]. In this sense, our results show that inhibition of GSK-3 blocks axon formation in hippocampal neurons, in agreement with previous results [31], without affecting the growth of other neurites. This inhibition is confirmed by molecular weight shift of tau-1 and the absence of MAP1B-P (SMI-31) staining in inhibited neurons. Similar results have been recently published, indicating that inhibition of GSK-3 with other specific inhibitor blocks axon growth in DRG neurons [42]. All these data are in agreement with the first report of Shi et al. [31]. The effect of GSK-3 inhibition that blocks axon formation and elongation is only observed when neurons are treated since the first day of culture. Treatments after the first 24 h, produces one axon with an extensive branching. This branching effect may be produce by partial inhibition of GSK-3. Indeed, a role for GSK3 activity in cargo delivery by kinesins has been proposed [43,44], and the balance between the active and inactive state of GSK-3 would decide the point of cargo delivery and the outgrowth of a ramification in the axon.

This partial inhibition could also affect in different ways different substrates of GSK-3 modifying the pattern of growth of the axon. For example, CRMP2, another microtubule associated protein that is a substrate of GSK3 [43,44], and overexpression of this protein in hippocampal neurons produces multiple axons [45]. However, there are contradictory results about how the regulation of CRMP-2 by GSK3 affects axon outgrowth and elongation. While the expression of a mutation in the GSK3 phosphorylation site produces multiple axons [43], transfection of a mutant CRMP-2 in which the site required to prime GSK3 phosphorylation is altered affects axonal growth [44]. These data highlight the complexity of CRMP-2 regulation in axon outgrowth and may reflect the complexity of GSK-3 regulation.

4.2. What influence does GSK3 exert on neuronal polarity?

Some controversial data have recently surfaced regarding the function of GSK3 in establishing an axon. While inhibition of GSK3 was initially proposed to impair axon formation [31], it was more recently suggested that such inhibition leads to the formation of multiple axons [30]. It was argued that the difference between these two effects could be due to the use of different inhibitors and time periods. More recent data, using a different GSK3 inhibitor, and the GSK3 peptide inhibitor, indicated that the inhibition of GSK3 impaired axonal elongation of DRG [42].

In this report, we have used four different inhibitors and we have analyzed how these influence axon formation and elongation in the period of time between the first and sixth day after plating. One of these inhibitors, AR-A014418, has been tested against other 27 kinases and it has been demonstrated its specificity for GSK-3 [33] and our results show that the inhibitory effect of all inhibitors is dose dependent. In this way, we have demonstrated that GSK3 activity is essential for a neurite (Stage 2) to extend as an axon (Stage 3), and consequently the formation of the axon initial segment (AIS) [12]. Upon release from inhibition, neurons are able to develop axons and only one axon, containing one AIS *per* neuron. Indeed, when GSK3 is inhibited after the neurons have been cultured for 3, 2 or even 1 day in culture, an axon is specified and only a single axon is extended by each neuron.

Our data are in agreement with the data presented by Shi et al. [31] and Kim et al. [42] and the fact that some results have pointed the formation of multiple [30,46] may have arisen due to the use of the Tau-1 antibody which is GSK3 sensitive. When GSK3 activity is inhibited, tau-1 immunoreactivity augments and lower molecular weight bands can be detected in parallel with an increase in somatodendritic tau staining. Moreover, on occasions a curling and branching of the axon occurs, which when passing close to the soma and dendrites can be mistaken as another axon. Alternatively many labs use other phosphoepitopes to label axons such as MAP1B-SMI 31. However, in this case no more accurate identification of the axons is facilitated, since SMI-31 is dependent on GSK3 phosphorylation.

Moreover, to avoid any epitope dependent or GSK3 related effects, we have taken advantage of the fact that certain proteins are specifically localized in the axon initial segment (AIS) of mature axons. This axonal domain appears in hippo-

campal neurons after axon formation and it can be identified using antibodies against voltage-gated sodium channels [12]. Thus, number of axons in a neuron can be identified by the number of AIS that form.

When we analyzed longer periods of GSK3 inhibition (6DIV), we found that 30% of neurons still did not extend an axon, while 10% developed a short and aberrant axon. Moreover, 48% of neurons extend an axon which we understand as an "escape" phenotype from pharmacological inhibition. Indeed, when we analyzed the population that developed an axon in more detail, the processes were initiated from a single axonal initial segment. Only a small proportion of the neurons developed multiple axons, which is not unusual as such circumstances were observed in control cultures (6% in untreated neurons versus 10% in neurons in which GSK3 was inhibited over 6-days). And more important, when inhibition was withdrawn after three days, a normal phenotype was almost completely recovered and 90% of the neurons contained a single AIS/neuron. This result permits conclude that independently of the different interpretations given to these neurites as potential multi-axons in GSK3-inhibited neurons, after withdrawn of inhibition only a single mature and functional axon is generated.

Consequently, our data show that although a certain amount of GSK3 activity is necessary during the first 24 h in culture for an axon to be established, the subsequent inhibition after this period does not appear to compromise axonal specification. However, the subsequent inhibition of GSK3 activity affects the final phenotype of the axon which is no longer completely normal, becoming more branched, arched and ramified than in normal cultured hippocampal neurons. To a certain extent, this agrees with data indicating that the inhibition of GSK3 promotes elongation mediated by neurotrophins in neurons of the peripheral nervous system [26]. All these data and previous studies [26,40] indicate that a certain amount of GSK3 activity is essential to specify an axon, and that maintaining axon extension does not require a major contribution of GSK3 activity. Finally, we can also conclude that axonal ramification is exacerbated by the inhibition of GSK3.

Most studies of the function of GSK3 in neuronal morphology and physiology have focused on the more abundant GSK3β, although some studies have highlighted a role for GSK3α in neuronal diseases [47]. Thus, we hypothesize that axon formation might depend on both isoforms; particularly since the GSK3 inhibitors used are unable to differentiate between the two of them.

We found that both GSK3 isoforms are present in the axon growth cone and that both are essential for axon specification. Hence, in our experiment with shRNAs, which strongly suppress specifically either GSK3α or GSK3β, any of them is enough to impair axon formation. This was confirmed using two different shRNAs for each isoform.

Thus, we can not rule out the possibility that each isoform may have a specific function in neuronal morphogenesis. In this sense, neurons were the GSK3β isoform was suppressed were not able to develop or initiate the growth of dendrites after 3DIV compare to neurons where only the GSK3α isoform was suppressed. Indeed, GSK3β plays an essential role in dendrite initiation and growth [48].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.03.018.

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